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SHORT COMMUNICATION

Results in Inhibition of Vaccinia Virus Replication

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Studies of interferon (IFN)-treated virus-infected animal cells have revealed the 2-5A system (2-5A synthetase/RNase L enzymes) as being responsible for virus inhibition only in the case of picornaviridae. To investigate whether those IFN-induced enzymes could be responsible for inhibition of poxvirus replication, we have generated recombinant vaccinia viruses (VV) containing the corresponding genes (VV-2-5AS and VV-RL, respectively). RNase L produced in cells infected with VV-RL leads to rRNA degradation and inhibition of virus protein synthesis, which correlates with about 92% reduction in virus yields by 48 hr after infection. Combined expression of this enzyme with 2-5A-synthetase further inhibits virus yields. The pattern of rRNA fragments produced by infection with viruses VV-RL and/or VV-2-5AS is the characteristic for activation of the 2-5A pathway by IFN treatment. Combined infection of VV-RL together with vesicular stomatitis virus (VSV) demonstrates this inhibition to be specific for VV and not due to a general effect. Breakdown of rRNA is largely due to the recombinant vector-derived enzyme, since a C-terminal deletion mutant of RNase L is inactive and the extent of rRNA degradation induced by infection with VV-RL is similar in cells treated or not with IFN. Moreover, the anti-VV effects of RNase L is also observed in a cell line lacking the endogenous ds RNA-dependent protein kinase (PKR). Thus, our findings provide direct evidence for antiviral activity of the 2-5A system on poxviruses.

Interferons (IFN) are cytokines released from animal cells in response to virus infection or to a variety of other stimuli. By binding to specific cell surface receptors, IFN exerts a wide range of functions, including inhibition of virus multiplication (*1, 2*). The varying effects of IFN are mediated by more than 30 different proteins which are induced after IFN treatment of the cells (*1–3*) and only the role of a few of them in the antiviral state is beginning to be elucidated. The best characterized IFN-induced proteins are the Mx family of proteins, the two double-stranded (ds) RNA-dependent enzymes, a 68-kDa protein kinase (PKR) and 2-5A-synthetase, and the 2-5A-dependent RNase (RNase L) (*3, 4*). The proteins 2-5A-synthetase and RNase L constitute, together with 2-5A-phosphodiesterase, what is known as 2-5A pathway or 2-5A system, an IFN-regulated RNA degradative pathway (*5*). Although basal levels of these proteins are found in most, if not all, mammalian cells, treatment with IFN induces at least four different forms of 2-5A-synthetase and a unique RNase L (reviewed in Ref. 3). Double-stranded RNA activates the 2-5A-synthetases that, in the presence of ATP, synthesize a complex mixture (referred to as 2-5A) of 5′-triphosphorylated oligoadenylic acid, ppp(A2′p5)nA, that binds to and activates endoribonuclease RNase L (*2*). Activated 2-5A-dependent RNase L cleaves viral and cellular RNAs, with the result of general inhibition of protein synthesis (*2*).

Characterization of the biological role of the 2-5A system has advanced by cloning of murine and human RNase L genes (*6*) which encode proteins of very interesting molecular architecture (*7, 8*). Human RNase L (83.5 kDa) contains nine ankyrin-like repeats at the N terminus which are immediately followed by a complete protein kinase domain and next to it, in the C terminus, the residues where the endoribonuclease activity is located. Ankyrin repeats have been involved in mediating intra- and intermolecular protein–protein interactions in many different types of proteins (*9*). Systematic biochemical studies of IFN-treated virus-infected cells has only revealed the 2-5A system as responsible for virus inhibition in the case of picornaviridae. Direct evidence for antiviral activity of this pathway was shown by constitutive expression of a cDNA encoding human 40-kDa 2-5A-synthetase (*10–12*). The inhibitory effect is selective for mengovirus and encephalomyocarditis (EMC) virus with little effect on either vesicular stomatitis virus (VSV) or herpes simplex 2 (HSV-2) (*10–12*). Inhibition of picornaviridae by IFN also requires functional RNase L as has

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been demonstrated in a stable cell line expressing high levels of a truncated RNase L which is a dominant negative mutant of this enzyme (*7*). However, the contribution of the 2-5A system to the antiviral activities of IFN almost certainly goes beyond the picornaviridae family of viruses and is obscured by virus-induced IFN-resistance mechanisms (reviewed in Refs. 2 and 3). There is evidence suggesting that this is probably the case for vaccinia virus (VV), a poxvirus which replicates in the cytoplasm of a wide range of cells, and that has been found to be resistant to IFN in many cultured cell lines (*13, 14*), while sensitive in others (*15*) and in experimental animals (*16*). The reason for the differential sensitivity of VV to IFN *in vivo* and *in vitro* might be related to the ability of the virus to synthesize interfering products. Indeed, VV displays an impressive variety of defensive strategies to evade the host response to infection, including counteraction of IFN by strategies at two different levels (reviewed in Ref. 17): production of soluble type I and II IFN receptors which blocks IFN action at a very early stage (*18, 19*) and modulation of the activity of the IFN-induced proteins. The product of ORF E3L competitively binds ds RNA and prevents the activation of the IFN-induced ds RNA-activated PKR (*20*) and probably also 2-5A-synthetase (*21*). Down regulation of the activation of PKR is also attained by the product of ORF K3L, a protein with sequence similarity to eukaryotic initiation factor 2α (eIF2α), that competitively binds PKR and therefore blocks inactivation by phosphorylation of eIF2α (22). Besides these proteins, there are results suggesting that VV uses additional mechanisms to evade IFN action. The product of ORF A18R, a mediator in virus RNA metabolism (*23*), and NPH-I, an enzyme involved in virus transcription (*24*), could be modulating the 2-5A system but if this happens by a direct or indirect mechanism remains to be determined. Finally, there are also results suggesting VV-induced ATPase and phosphatase activities being involved in viral interference with the 2-5A system (*25, 26*).

In this investigation we have examined whether the IFN-induced enzymes 2-5A-synthetase and 2-5A-dependent RNase L could be responsible for inhibition of VV replication in situations of virus sensitivity to IFN. To this aim, we have generated VV recombinants able to express individually each one of the IFN-induced enzymes in the 2-5A pathway and analyzed the effect of their expression in VV replication, using a cell line where the virus is otherwise mainly resistant to IFN action. A recombinant virus where expression of RNase L can be regulated was obtained by cloning of the human gene, contained in plasmid ZC-5 (*6*), under the control of the bacteriophage T7 promoter into VV insertion vector pTM1-E (*27*) (Fig. 1A). We first generated plasmid pPR35-RL by insertion of a *Hin*dIII fragment of ZC-5 into the *Sma*I site of pPR35 (*28*). Plasmid pRSET-RL for expression in *Escherichia coli* was then obtained by cloning of a *Pst*I + *Kpn*I fragment of pPR35-RL into pRSET-B digested in the same way. It contains the coding sequence for RNase L, lacking only nucleotides coding for the 21 amino acids at the N terminus, fused to a sequence for 38 new amino acids that includes a stretch of 6 histidine residues and the 11 amino acid gene 10 leader peptide. We also generated plasmid pRSET-ΔN by *Nco*I digestion of pRSET-RL and religation. This plasmid is similar to pRSET-RL but lacking sequences coding for amino acids 228 to 741 of RNase L. Insertion plasmids pTM-RL and pTM-ΔN were then obtained by cloning DNA fragments from plasmids pRSET-RL (*Nde*I) and pRSET-ΔN (*Nde*I + *Nco*I), respectively, into the *Sma*I site of VV insertion vector pTM1-E (*27*) and they contain 8 extra amino acids in the N terminus in front of the initiation codon provided by pRSET-B. Recombinant viruses VV-RL and VV-ΔN were generated by homologous recombination of plasmids pTM-RL and pTM-ΔN into thymidine kinase (tk) gene of wild-type vaccinia virus (WR) and selection in human TK$^-$ 143B cells with 5′-bromodeoxyuridine (25 μg/ml) basically as described (*29*). Two rounds of plaque purification yielded homogeneous virus preparations containing the desired genes, as confirmed by Southern blot DNA hybridization analysis (data not shown). We also prepared a rabbit polyclonal anti-RNase L serum able to recognize proteins produced by these recombinant viruses, using the C-terminus of this protein (amino acids 340 to 741) expressed in *E. coli* as the antigen. Exponentially growing *E. coli* BL21 (DE3) cells carrying plasmid pRSET-Δ2S, generated by *Sac*I digestion of pRSET-RL and religation, were induced with 1 m*M* isopropyl-β-D-thiogalactopyranoside. The approximately 45-kDa induced protein, containing a polyhistidine metal-binding domain, was purified by using a nickel-charged sepharose resin (Invitrogen) in denaturing conditions. Binding was performed in 6 *M* guanidine hydrochloride, 0.1 *M* Na-phosphate, 0.01 *M* Tris/HCl, pH 8.0, 10 m*M* imidazole, and elution in 6 *M* urea, 0.5 *M* NaCl, 20 m*M* Na-phosphate, pH 7.8, 0.5 *M* imidazole. The protein was precipitated with TCA, the pellet thoroughly washed in acetone and, once dried, suspended in PBS and used as the antigen for injection into New Zealand white rabbits. Cell extracts obtained from mock-infected monkey BSC-40 cells or from cells infected for 24 hr with VV-RL or VV-ΔN were therefore analyzed by immunoblotting using this sera (Fig. 1B). Proteins of sizes expected for RNase L (lane 5) and C terminus deleted RNase L (RNase L-ΔN) (lane 6) were only obtained by coinfection with a second recombinant virus that contains a T7 polymerase gene under the control of virus constitutive promoter p7.5 (vTF7-3, herein vT7) (*30*). It is noticeable that for RNase L-ΔN, besides a main band of the expected molecular weight for the truncated protein, there are accompanying products of higher and lower molecular weights. Similar results were obtained

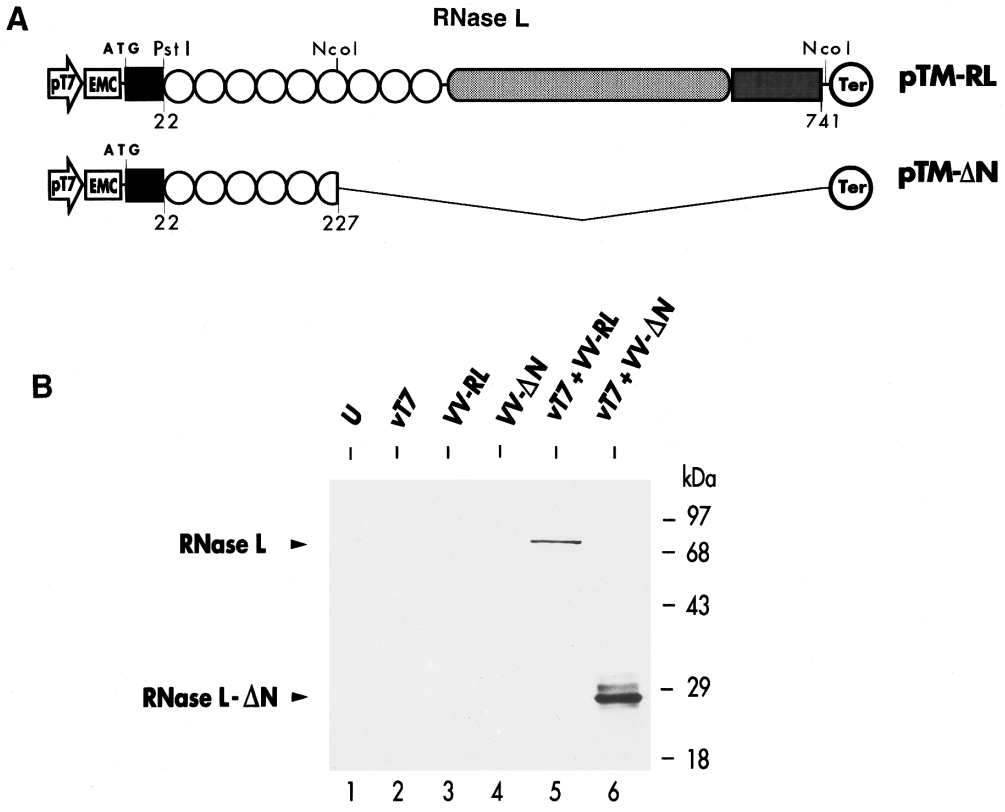

FIG. 1. Inducible expression of nearly full-length and truncated RNase L by recombinant vaccinia viruses. (A) Diagram showing the structure of the transfected genes pTM-RL and pTM-ΔN. pT7, bacteriophage T7 polymerase promoter; EMC, encephalomyocarditis virus sequences for cap-independent translation; solid boxes, pRSET-B sequences comprising an ATG, 6 histidine residues, and the gene 10 leader peptide; open circles, ankyrin-like repeats in 2-5A-dependent RNase L; gray oval, protein kinase homology region; gray rectangle, region required for RNase activity; Ter, T7 transcriptional terminator. (B) Expression of the recombinant proteins. Monolayers of BSC-40 cells were either mock-infected (lane 1), single-infected with VV-RL, VV-ΔN, or vT7 (m.o.i. 4) (lanes 2–4), or double-infected with vT7 + VV-RL (lane 5) or vT7 + VV-ΔN (lane 6) (m.o.i. 2 each virus) for 24 hr. Cell extracts (50 μg) were fractionated in SDS–10% polyacrylamide gels, transferred to nitrocellulose, and analyzed by immunoperoxidase staining after reactivity with rabbit polyclonal serum against RNase L.

using T7-tag, a commercial monoclonal antibody directed against the gene 10 leader peptide in the N terminus (data not shown). These bands might reflect complex conformation of RNase L-ΔN due to intramolecular interaction of the ankyrin repeats in the protein. Endogenous RNase L could not be detected in mock-infected BCS-40 cells (Fig. 1B, lane 1), probably because our polyclonal antibodies prepared against the human protein are not able to recognize monkey RNase L.

Late during VV infection of cells in culture, concomitant with synthesis of complementary mRNAs due to symmetrical transcription of late genes, 2-5A is produced at high levels (*14, 26*) and although it is biologically active when assayed *in vitro* it is not able to inhibit VV replication in cultured cells (*14*). The resistance of VV to the 2-5A system is probably due to the existence of viral products that directly or indirectly counteract the activation of endogenous RNase L. The levels of activatable RNase L and of 2-5A might be critical in the sensitivity/resistance phenomenon of VV to the 2-5A system. To address this hypothesis, we measured RNA stability in BSC-40 cells infected with vT7 and VV-RL at different times of infection. Total RNA was extracted from mock-infected cells or from cells infected either with vT7 (m.o.i. 4) or doubly infected with vT7 + VV-ΔN or vT7 + VV-RL (m.o.i. 2 each virus) for the indicated periods of time (Fig. 2A) and was analyzed by electrophoresis on formaldehyde agarose gels. Interestingly, in cells doubly infected with vT7 + VV-RL for 24 hr, expression of RNase L induces breakdown of RNA as seen by rRNA cleavage (lane 5). Cleavage of rRNA required the expression of RNase L and was not observed in cells infected with vT7 (lane 2) and only to a very low extent in cells expressing RNase L-ΔN (lane 7). Because recombinant RNase L produced in insect cells has been shown to dimerize upon binding to 2-5A (*31*), we cannot exclude that RNase L-ΔN expression might cause minimum activation of endogenous RNase L. Time-course experiments with vT7 + VV-RL showed that rRNA cleavage starts at about 14 hr after infection (lane 4), coincident with the time when RNase L is first detected by immunoblotting with sera against RNase L (Fig. 2B). Discrete rRNA fragments characteristic of activation of the 2-5A/RNase L pathway (*32, 33*) are detected up to 24 hr of infection (Fig. 2A, lanes 4 and 5).

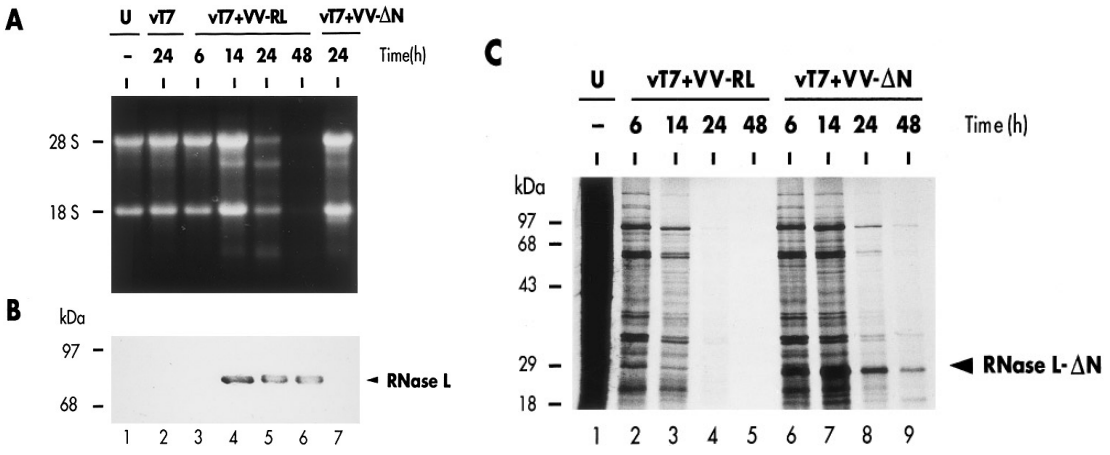

FIG. 2. Induced RNase L cleaves rRNA and inhibits virus protein synthesis in BSC-40 cells. (A) Time-course of RNA degradation. Monolayers of BSC-40 cells were either mock-infected (lane 1), single-infected with vT7 (m.o.i. 4) (lane 2), or double-infected with vT7 + VV-RL (lanes 3–6) or vT7 + VV-ΔN (lane 7) (m.o.i. 2 each virus). Control infections proceeded for 24 hr (lanes 2 and 7) or for the indicated times in the case of vT7 + VV-RL (lanes 3–6). Total RNA was purified using Ultraspec-II RNA Resin Purification System (Biotecx), and the amount of RNA corresponding to 3×10^5 cells was fractionated in 1% agarose-formaldehyde gels and stained with ethidium bromide. Abundant RNAs, 28 S and 18 S rRNA, are indicated. (B) Time-course of RNase L synthesis. Cells were either mock-infected or infected as before. Cell extracts (50 μg) were fractionated in SDS–10% polyacrylamide gels, transferred to nitrocellulose, and immunoblotted using rabbit polyclonal antiserum against RNase L as described. (C) Time-course effect of RNase L on *de novo* protein synthesis. Monolayers were mock-infected or infected with vT7 + VV-RL or vT7 + VV-ΔN (m.o.i. 5 each virus) for the indicated times. Cells were labeled for 15 min with ^{35}S-labeled methionine + cysteine (100 μCi/ml) and protein extracts (50 μg) were fractionated in SDS–polyacrylamide gels and autoradiographed. Lane 1 is over-exposed reflecting lack of shut-off in protein synthesis in mock-infected cells compared to infected cells (lanes 2–9).

Very little rRNA is detected by 48 hr (Fig. 2A, lane 6), suggesting further degradation of RNA fragments first produced by this or other RNases. Clearly, the results of Fig. 2 demonstrate that RNase L induced from a recombinant VV causes extensive rRNA degradation, in spite of the synthesis of viral interfering products.

Degradation of RNA observed in cells that express RNase L might have as a consequence the inhibition of virus protein synthesis. Thus, we performed a time-course experiment where, due to shut-off of cellular protein synthesis as a consequence of infection, we measured *de novo* synthesis of viral proteins (Fig. 2C). Monolayers of BSC-40 cells were either mock-infected (lane 1) or infected with vT7 + VV-RL (lanes 2–5) or vT7 + VV-ΔN (lanes 6–9) using the same multiplicity for the indicated times. Then, cells were pulse-labeled with [^{35}S]-methionine + cysteine (100 μCi/ml) in methionine and cysteine-free media for 15 min. After washing with PBS, cells were collected in lysis buffer, protein extracts were fractionated in SDS–polyacrylamide gels, and, once dried, gels were autoradiographed. Inhibition of virus protein synthesis by RNase L is clearly observed starting at about 14 hr after infection (compare lanes 3 and 7) when *de novo* protein synthesis is maximum (compare lanes 6–9). After 24 hr of infection, low protein synthesis activity is found in control infections (lanes 8 and 9) but inhibition by RNase L can still be observed (lanes 4 and 5). A very abundant protein of size expected for RNase L-ΔN is produced in cells infected with vT7 + VV-ΔN, while levels of full-length RNase L synthesized are probably very low (compare lanes 7 and 3). These findings provide direct evidence that degradation of RNA induced throughout expression of RNase L by a recombinant VV results in inhibition of virus protein synthesis.

We next examined if by increasing the levels of 2-5A through expression of 2-5A-synthetase in combination with RNase L, further enhances the inhibition of VV protein synthesis and what is the net effect in viral replication. To this aim we generated VV recombinant VV-2-5AS where expression of human 40-kDa 2-5A-synthetase is driven by the virus constitutive early-late promoter p7.5. The recombinant was obtained by homologous recombination of plasmid pSC-2-5AS into wild-type virus (WR) thymidine kinase (tk) gene basically as described (*29*). The DNA was obtained by cloning a 1.2-kb fragment from plasmid pTL4-2-5AS (*Nco*I + *Xho*I digested and ends filled with Klenow fragment) into the *Sma*I site of VV insertion vector pSC 11 (*34*). It contains the complete human 40-kDa 2-5A-synthetase coding sequence, lacking the methionine residue at the N terminus, and the *E. coli* β-galactosidase gene (*lac Z*) under the control of a late viral promoter (p11). Recombinant viruses were selected in human TK$^-$ 143B cells using X gal and 5′-bromodeoxyuridine (25 μg/ml) (*29*), and two rounds of plaque purification yielded homogeneous virus preparations. Monkey BSC-40 cells were then infected with different combinations of vT7, VV-RL, VV-ΔN, or VV-2-5AS using 5 PFU/cell of each virus. All cultures received a total virus multiplicity of 15 PFU/cell using an auxiliary recombinant virus VV-LUC (*35*), which as other recombinants used in this work is a tk(−) virus. We have compared the stability of rRNA at two different times of infec-

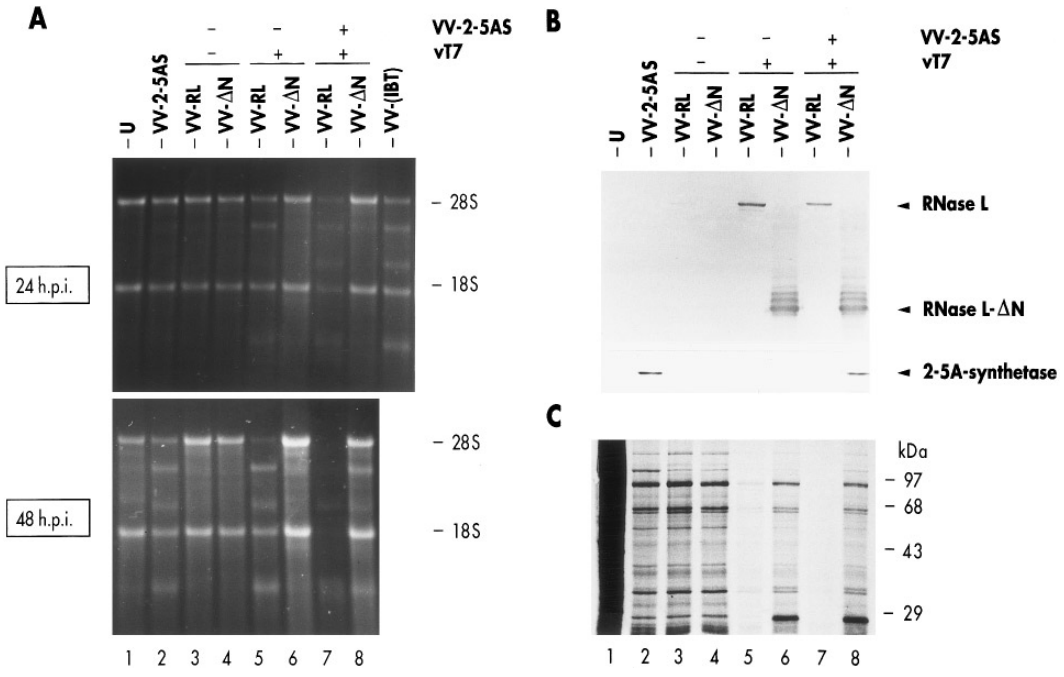

FIG. 3. Expression of 2-5A-synthetase together with RNase L increases effects on RNA stability and protein synthesis. (A) Analysis of RNA degradation. Monolayers were either mock-infected (lane 1) or infected with indicated combinations of viruses vT7, VV-RL, VV-ΔN, and VV-2-5AS (m.o.i. 5 each virus) (lanes 2–8) for 24 hr (upper panel) or 48 hr (lower panel). Total multiplicity was maintained 15 at all times using VV-LUC when required. Cells were also treated with IBT (15 μM) and infected with wild-type virus (VV) for 24 hr (m.o.i. 15) (lane VV-(IBT)). Total RNA was purified as before, and the amount of RNA corresponding to 3×10^5 cells was fractionated in 1% agarose-formaldehyde gels and stained with ethidium bromide. Abundant RNAs, 28 S and 18 S rRNA, are indicated. (B) Synthesis of recombinant proteins. Monolayers were infected for 48 hr as before, cell extracts (50 μg) were fractionated in SDS–10% polyacrylamide gels, transferred to nitrocellulose, and immunoblotted using rabbit polyclonal antiserum specific for RNase L (upper panel) or 2-5A-synthetase (lower panel). (C) *De novo* protein synthesis. Monolayers were infected for 24 hr as before and labeled with ^{35}S-labeled methionine + cysteine (100 μCi/ml) for 15 min as described. Protein extracts (50 μg) were fractionated and dried gels were autoradiographed. Lane 1 is over-exposed reflecting lack of shut-off in protein synthesis in mock-infected cells compared to infected cells (lanes 2–8).

tion (24 and 48 hr), with levels of expression of the recombinant proteins (RNase L and 2-5A-synthetase), *de novo* synthesis of virus proteins, and virus yields. As shown, cleavage of rRNA is observed at 24 hr of infection with vT7 + VV-RL (Fig. 3A, lane 5), but degradation increases upon addition of VV-2-5AS (compare lanes 7 to 5). Control experiments using VV-ΔN instead of VV-RL demonstrate that lack of RNA stability is due to expression of nearly full-length RNase L (compare lanes 6 and 8 to 5 and 7, respectively). At 48 hr, in cells infected with vT7 + VV-RL or vT7 + VV-RL + VV-2-5AS, rRNA degradation progresses (Fig. 3A, compare lanes 5 and 7 in upper and lower panels) and for triple infections most rRNA is degraded by this time of infection. Interestingly, expression of 2-5A-synthetase by itself induces some rRNA cleavage at 48 hr after infection (lane 2). This is probably due to low levels of activation of endogenous RNase L by the high levels of 2-5A oligos produced by 2-5A-synthetase expressed by the recombinant virus. Activation of endogenous RNase L might be also the cause of low levels of rRNA degradation observed in triple infection vT7 + VV-ΔN + VV-2-5AS at 48 hr of infection (lane 8). Noteworthy, the pattern of rRNA fragments produced by infection with recombinant viruses expressing RNase L and/or 2-5A-synthetase is similar to the one obtained in cells infected for 24 hr with wild-type virus (m.o.i. 15) in the presence of isatin-β-thiosemicarbazone (IBT) (15 μM) added to the culture after viral adsorption and maintained for the duration of the experiment (*23*) (Fig. 3A, upper panel, lane VV-(IBT)). This is an antipoxviral drug which causes a significant increase in the amount of ds RNA synthesized during VV infection, therefore inducing cleavage of rRNA with a pattern that is the characteristic one for activation of the 2-5A pathway (*36*). The levels of recombinant proteins RNase L and 2-5A-synthetase were analyzed by using specific rabbit polyclonal sera and the results obtained at 48 hr of infection are presented in Fig. 3B. Cleavage of rRNA correlates in all cases with expression of RNase L and/or 2-5A-synthetase (compare lower panel in Fig. 3A and 3B). Samples presenting the highest levels of rRNA degradation also show clear signs of protein synthesis inhibition with amounts of RNase L and 2-5A-synthetase being, respectively, moderate and severely inhibited. Analysis of *de novo* virus protein synthesis at 24 h of infection (Fig. 3C) showed very low levels of protein synthesis activity in cells infected with vT7 + VV-RL that are further reduced by coexpression of 2-5A-synthetase (compare lanes 5 and 7).

TABLE 1

Viral Yields in Cells Infected with Recombinant Viruses Expressing RNase L and/or 2-5A-Synthetase

	24 hr p.i.		48 hr p.i.	
	PFU/ml	Percentage inhibition	PFU/ml	Percentage inhibition
VV-RL	2.4×10^6 (0.3)	—	2.4×10^7 (0.5)	—
VV-2-5AS	9.6×10^5 (1.2)	60.0	5.4×10^6 (1.1)	77.5
vT7 + VV-RL	6.0×10^5 (1.9)	75.0	1.9×10^6 (0.3)	92.1
vT7 + VV-RL + VV-2-5AS	5.7×10^5 (1.9)	76.3	8.1×10^5 (0.3)	96.6

Note. Monolayers of BSC-40 cells grown in 24-well plates were infected with viruses vT7, VV-RL, and VV-2-5AS (m.o.i. 5 each virus). Total multiplicity was maintained at 15 at all times, using VV-LUC when required. Cells were collected in media at 24 and 48 hr of infection, freeze-thaw three times, sonicated, and virus yields titrated on BSC-40 cells. The results are mean values of three independent experiments and standard deviations are given in parenthesis.

The effect of expression of enzymes in the 2-5A pathway on virus yields is shown in Table 1. Production of infectious virus at 24 and 48 hr was quantitated by plaque assay and values referred to results obtained for cells infected with VV-RL in the absence of vT7. Expression of 2-5A-synthetase by itself causes inhibition of virus replication by 60 and 77.5% at 24 and 48 hr of infection, respectively. Expression of RNase L inhibits virus yields by 75 and 92.1% at 24 and 48 hr of infection, respectively, while combined expression of RNase L and 2-5A-synthetase enhances the inhibition of virus growth to 96.6% at 48 hr. Reduction in virus yields due to RNase L expression is a late event and it is only apparent after 12 hr of infection (data not shown). The limited reduction in virus yields observed in these experiments is probably due to the kinetics of RNase L activation. Synthesis of this protein is detected for the first time 14 hr after infection (Fig. 2A, lane 4) and inhibition of protein synthesis is clearly observed starting at about the same time (Fig. 2C, lane 3), a relatively late time point in VV infection when virus assembly has been mainly completed. Therefore, the one log reduction observed in our expression system could be an underestimate of the potency of the 2-5A system in VV sensitivity to IFN, or it may represent the maximum effects.

In order to establish that inhibition of VV replication is specific, we have also analyzed the effect of RNase L expression in the replication of vesicular stomatitis virus (VSV), which is very sensitive to IFN treatment, although it has been previously described as resistant to the 2-5A system (*12*). Monolayers of BSC-40 cells were infected with VSV (m.o.i. 10), alone or in combination with either wild-type VV (m.o.i. 10) or recombinant viruses: vT7 (m.o.i. 5) + VV-RL (m.o.i. 5) or vT7 (m.o.i. 5) + VV-ΔN (m.o.i. 5) and VSV viral yields were established at different times of infection. Production of active RNase L, demonstrated by protein analysis (data not shown), has no effect on VSV replication as indicated in Table 2.

Because there is endogenous RNase L in the cells used in these experiments, we have tried to separate the contribution of endogenous and recombinant enzymes by using stable cell line SVT2/ZB1.4 expressing a dominant negative mutant of RNase L (*7*). However, infection of these cells with vT7 + VV-RL produced degradation of rRNA similar to control cells SVT2/pSVL. The most likely explanation is that the levels of mutant RNase L, which has been described as insufficient to counteract the anti-EMCV effect of IFN (*7*), are below the amounts of RNase L produced by vT7 + VV-RL infection. To further attempt to separate the effects of activation of endogenous cellular RNase L from those effects due to activation of the recombinant VV vector derived enzyme, we compared RNA stability in BSC-40 cells treated for 18 hr with IFN (1000 international units [U] per ml of human lymphoblastoid IFN-α, 1×10^8 U/μg, Wellcome Res.) and then infected with vT7 (m.o.i. 4) or doubly infected with vT7 + VV-ΔN or vT7 + VV-RL (m.o.i. 2 each virus) for 24 hr. In these cells VV replication is nearly resistant to IFN treatment with only a 35% reduction in virus yields at 24 hr and the levels of RNase L are increased by IFN

TABLE 2

Effect of RNase L Expression on VSV Replication

	VSV	VSV + VV	VSV + vT7 + VV-RL	VSV + vT7 + VV-ΔN
6 hr p.i.	5.0×10^5	3.0×10^5	3.0×10^5	4.0×10^5
14 hr p.i.	9.0×10^5	7.0×10^5	1.2×10^6	1.2×10^8
24 hr p.i.	1.1×10^6	8.0×10^5	9.0×10^5	9.0×10^5

Note. Monolayers of BSC-40 cells were either mock-infected or infected with wild-type VV (m.o.i. 10) or recombinants: vT7 (m.o.i. 5) + VV-RL (m.o.i. 5) or vT7 (m.o.i. 5) + VV-ΔN (m.o.i. 5). After 30 min of adsorption, VSV (m.o.i. 10) was also added. Following another 30 min the virus inoculum was removed, cells were washed, and medium containing 2% NCS was added. Infections were also performed with VSV or vaccinia viruses alone as controls. Cell supernatants were collected at the indicated times of infection and VSV virus yields titrated on BSC-40 cells for 14 hr. The results are presented in PFU/ml.

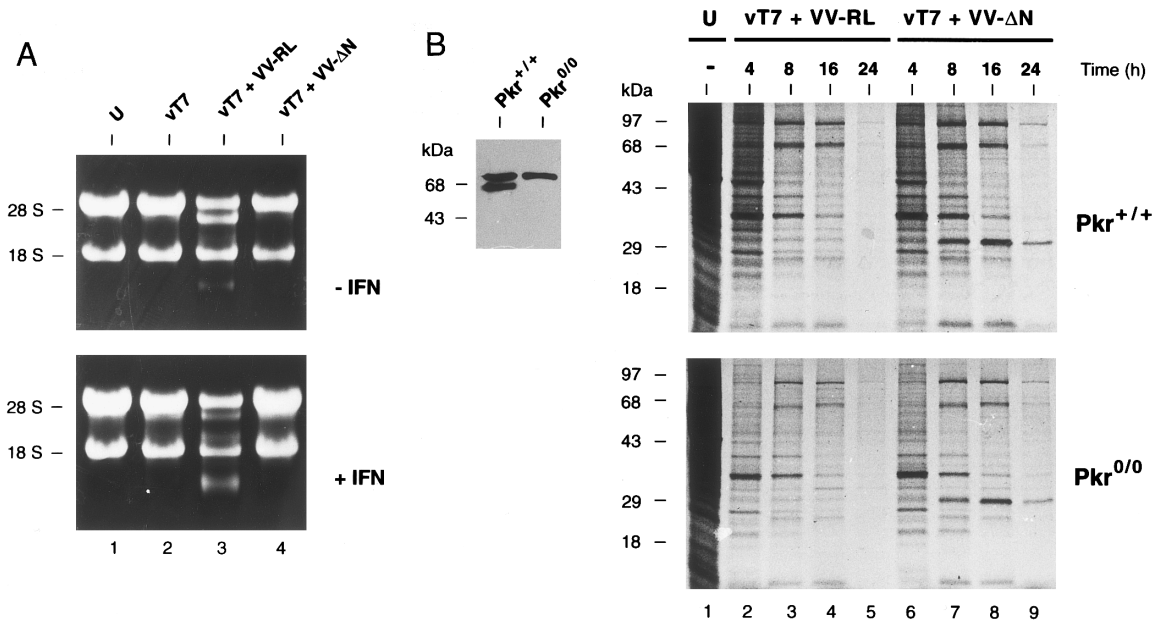

FIG. 4. Effects produced by RNase L induced by the recombinant vector are not dependent on other IFN-induced enzymes. (A) Analysis of RNA degradation in IFN-treated cells. Monolayers of BSC-40 cells were treated for 18 hr with 1000 international units [U] per milliliter of human lymphoblastoid IFN-α (lower panel) or left untreated (upper panel). Cells were either mock-infected (lane 1), single-infected with vT7 (m.o.i. 4) (lane 2), or double-infected with vT7 + VV-RL (lane 3) or vT7 + VV-ΔN (lane 4) (m.o.i. 2 each virus) for 24 hr. Total RNA was purified as before, and the amount of RNA corresponding to 3×10^5 cells was fractionated in 1% agarose-formaldehyde gels and stained with ethidium bromide. Abundant RNAs, 28 S and 18 S rRNA, are indicated. (B) Effect of RNase L on *de novo* protein synthesis in cells devoid of PKR. Left panel, immunoblot of protein extracts prepared from 3T3-like cells derived from homozygous PKR knockout mice (*Pkr*$^{0/0}$) or wild-type animals (*Pkr*$^{+/+}$) using a rabbit polyclonal antiserum specific for mouse PKR. Right panels, monolayers of Pkr +/+ (upper panel) or *Pkr*$^{0/0}$ (lower panel) were mock-infected (lane 1) or infected with vT7 + VV-RL (lanes 2–5) or vT7 + VV-ΔN (lanes 6–9) (m.o.i. 10 each virus) for the indicated times. Cells were labeled as before and protein extracts (50 μg) were fractionated in SDS–polyacrylamide gels and autoradiographed.

(*13, 26*). As shown in Fig. 4A, in cells pretreated with IFN, cleavage of rRNA could not be observed in vT7 or vT7 + VV-ΔN infections (lanes 2 and 4). In cells expressing recombinant RNase L, the extent of rRNA degradation was only slightly increased by IFN treatment (compare lanes 3). Therefore, RNase L induced by the recombinant virus VV-RL, and not the endogenous enzyme, is responsible for rRNA cleavage. Because activation of endogenous PKR could also contribute to inhibition of VV replication, we tested the anti-VV effect of recombinant RNase L in cells lacking PKR. Mouse cell lines derived from homozygous PKR knockout mice (*Pkr*$^{0/0}$) or wild-type animals (*Pkr*$^{+/+}$) (*37*) (Fig. 4B) were infected with VV recombinants and the levels of proteins and virus yields were evaluated. Absence of murine PKR in the cell line derived from *Pkr*$^{0/0}$ mice is shown by immunoblot in Fig. 4B. Pulse-labeling and SDS–PAGE analysis showed that the inhibition of virus protein synthesis by RNase L was comparable in cells with or without PKR and was clearly more pronounced than in cells expressing inactive RNase L (Fig. 4B). As a consequence of inhibition of protein synthesis by RNase L there is at 24 hr of infection a reduction in virus yields, referred as in Table 1, of 63% for *Pkr* $^{0/0}$ and 84% for *Pkr*$^{+/+}$ cells. The results of Fig. 4 provide additional evidence that expression of recombinant RNase L by VV-RL, and not the endogenous enzyme, is responsible for inhibition of VV replication and that endogenous PKR does not contribute significantly to the anti-VV effects of RNase L.

The results presented in this investigation provide direct evidence for an anti-VV role of ds RNA-dependent 2-5A-synthetase and 2-5A-dependent RNase L, key enzymes in the molecular mechanisms of IFN action. By increasing the levels of these enzymes, coupled with their ability to inhibit protein synthesis, we have been able to overcome the factors that VV uses to evade the IFN action. Activation of the 2-5A-pathway induced by infection with VV-2-5AS or VV-RL alone most probably requires the participation of endogenous enzymes: RNase L that becomes active upon increased levels of 2-5A are produced by infection with VV-2-5AS, or 2-5A synthetase causing accumulation of 2-5A (*14, 26*) that activate the increased levels of RNase L achieved by VV-RL. Basal levels of enzymes in the 2-5A system are present in variable amounts in most if not all mammalian cells. RNase L is a low abundance protein which is increased from 2- to 10-fold in some cells after treatment with IFN (*38*). On the contrary, the amount of 2-5A-synthetase usually increases substantially in response to IFN (10- to 10,000-fold) although the high levels of 2-5A-synthetase produced in cells transfected with the 40-kDa 2-5A-synthetase human gene are not sufficient by them-

selves to protect against picornaviruses, and minimum levels of RNase L are also required (*39*). These data can be interpreted to mean that the antiviral state in the cell is compatible with amounts of 2-5A-synthetase in a very broad range while small changes in levels of activated RNase L are really critical. Our results, obtained in cells infected with the recombinant viruses, provide evidence for this hypothesis and showed that expression of RNase L by itself causes greater inhibition of VV replication than expression of 2-5A-synthetase (Table 1).

The VV cell system described here for expression of enzymes in the 2-5A pathway has two main advantages: first, it makes it possible to increase the levels of these enzymes in a controlled fashion, and second, virus infection provides at the same time a very powerful activator of the system, i.e., dsRNA, a byproduct of virus replication. We believe that RNase L activation requires first of all production of the protein and then synthesis of ds RNA, the activator of endogenous 2-5A-synthetase that produces 2-5A oligonucleotides at late times of infection (*14, 25, 26*). We have previously described that 2-5A levels in untreated BSC-40 cells are 1.4 n*M* by 8 hr of infection with VV and increase to 3.0 n*M* by 24 hr of infection (*26*). Low concentrations of 2-5A ($\leqslant$1 n*M*) are normally required to activate the 2-5A-dependent RNase *in vitro* (*14*) and 2-5A extracted from VV-infected cells is biologically active when assayed *in vitro* (*14*). Because degradation of RNA occurs during infection with VV-RL, our results strongly suggest that 2-5A accumulated in infected cells is also biologically active *in vivo.* Then, how can we explain that 2-5A produced during wild-type VV infection of BSC-40 cells does not induce rRNA breakdown? We propose the existence of a mechanism directly responsible of interference with 2-5A-dependent RNase L. Therefore, during infection of cells with the recombinant virus expressing RNase L, activation of this enzyme would be fulfilled only when enough levels of RNase L and its specific activator dsRNA accumulate as to outnumber viral-induced resistance mechanisms specific for this enzyme. Indeed, induced expression of PKR with a recombinant VV similar to VV-RL has been shown to counteract viral mechanisms of interference with this pathway and cause inhibition of its own replication (*40*). We envisage resistance or sensitivity of VV to IFN as a tightly regulated equilibrium between levels of the IFN-induceable proteins (basal, IFN-induced, or recombinant virus-induced enzymes in our expression system), their activators, such as dsRNA and 2-5A provided upon viral infection, and virus-encoded resistance products. Subtle changes in the levels of some of the components in the 2-5A system can direct the cell toward an IFN-sensitive or -resistant phenotype that might also explain differences in IFN sensitivity found for VV infecting different cell lines (*13–15*). Although in our expression system several positive or negative regulatory factors are involved, maintaining the multiplicity of infection equal in each experiment with auxiliary recombinant tk(−) viruses warranties the same contribution of all these factors. Therefore, effects observed on rRNA breakdown can be specifically attributed to the vector-derived enzyme and not to quantitative differences in the levels of viral ds RNA produced.

In conclusion, in this report we provide direct evidence with animal cells in culture that by increasing the levels of enzymes in the 2-5A pathway we generate an antiviral state in the cell that is effective upon VV replication but does not affect VSV, which has been previously shown to be insensitive to this pathway. Since replication of VV is severely inhibited in IFN-treated animals, the biological significance of our findings is to point out that *in vivo* the 2-5A system is likely to be a mediator of the antipoxvirus action of IFN and that modulation of the 2-5A pathway might have therapeutic value. The unique system described here provides the means to search for virus interference factors throughout their interaction with RNase L. In addition, by expressing mutant or truncated forms of enzymes in the 2-5A system, it should be possible to define *in vivo* functional domains in these important proteins.

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